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Enzymatic staining of sialidase and β-galactosidase in polyacrylamide gel using chromogenic and fluorigenic substrates

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Abstract

Lysosomal sialidase is a membrane-bound enzyme, which found to be a complex with β-galactosidase and carboxypeptidase A. Due to its low stability a simple detection in polyacrylamide gels using chromogenic substrate was developed to speed up exploring this enzyme. *Vibrio cholera* sialidase and *Escherichia coli* β-galactosidase were used as a model. In a polyacrylamide gels using SDS-PAGE under non-reducing condition, bands showing enzyme activities of sialidase and β-galactosidase were detected following staining with the chromogenic substrates, 5-bromo-4-chloro-3-indolyl-α-D-*N*-acetylneuraminic acid (X-Neu5Ac) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) respectively. With this staining method, protein band corresponds to the enzyme activities could be located and detected as a permanently sharp blue band, which could be very useful to detect the protein band correspond to the enzyme activities even from partially purified enzyme. Detection of the *Vibrio cholera* sialidase and *Escherichia coli* β-galactosidase using fluorigenic substrates, sialyl-methylumbelliferyl α-glycoside (MU-Neu5Ac) and galactosyl-methylumbelliferyl β-glycoside, respectively, were used as comparison.

Keywords: X-Neu5Ac; X-Gal; sialidase; β-galactosidase; SDS-PAGE.

Introduction

Sialidase (EC 3.2.1.18) is a hydrolytic enzyme that releases sialic acid, which usually bound through α-2,3, α-2,6, or α-2,8 linkages to oligosaccharides or glycolipids (Traving and Schauer, 1998). Sialic acids are relatively big, hydrophilic molecules possessing a negative charge, so they have been proposed to be involved in numerous biological and physicochemical functions (Schauer, 1985). In mammals sialidases have been found in various cellular locations, either as cytosolic or membrane-bound enzymes (e.g. lysosomes, Golgi, plasma membrane and nuclear membrane) (Traving and Schauer, 1998). The study of membrane-bound sialidases is fraugth with difficulties, due to their low stability and their occurrence in a complex with other enzymes such as acid β-galactosidase (EC 3.2.1.23) and carboxypeptidase A (EC 3.4.16.1) (Hiraiwa *et al*., 1996; Hiraiwa *et al*., 1997; Nagaoka *et al*., 1998; Verheijen *et al*., 1985).

An enzyme-specific staining method for sialidase with fluorigenic substrate, sialyl-methylumbelliferyl α-glycoside (MU-Neu5Ac) has been already described (Berg *et al*., 1985) and applied (Byers *et al*., 2000; Samollow *et al*., 1985). This method can be simply and rapidly performed to detect protein band correspond to the enzyme activity in polyacrylamide gel following electrophoresis. Using fluorigenic substrate, galactosyl-methylumbelliferyl β-glycoside (MU-Gal), β-galactosidase activity in polyacrylamide gel can be also detected with the same method. However, since the fluorescence of the protein band, which showing enzyme activity, usually shows a spread fluorescence, it is sometimes difficult to

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know the real protein band correspond to the enzyme activity from partially purified enzyme. On the other hand, chromogenic substrates, X-Neu5Ac (Fujii *et al*., 1993) and X-Gal (Horwitz *et al*., 1964) are routinely used to select bacterial colonies expressing sialidase and β-galactosidase, respectively. The X-Gal was also used for histochemical detection of β-galactosidase (Weiss *et al*., 1997; Weiss *et al*., 1999). The chromogenic substrate for phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), is largely used for immunoblotting (Wolf *et al*., 1968c), immunohistochemistry (Wolf *et al*., 1973) or detection of alkaline phosphatase in disk electrophoresis (Epstein *et al*, 1967; Epstein *et al*., 1968). Another enzyme reactions using this chromogenic substrate for histochemical reaction were also reported for sulfatase (Wolf *et al*., 1967), β-xylosidase (Wolf *et al*., 1968a), ribonuclease (Wolf *et al*., 1968b).

Detection of protein band in a polyacrylamide gel showing sialidase and β-galactosidase activity, which could be used for the detection of membrane-bound sialidase even from partial purification step would be very useful to speed up the study about this enzyme. In this study we demonstrated detection of protein band correspond to sialidase and β-galactosidase activity using X-Neu5Ac and X-Gal, respectively, in polyacrylamide gels following SDS-PAGE under non-reducing condition.

Materials and Methods

Materials

Electrophoretic-grade acrylamide, *N,N'*-methylenebisacrylamide was obtained from BioRad (Munich, Germany). *N,N,N',N'*-tetramethylenediamine, galacto-methylumbelliferyl β-glycoside (MU-Gal) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and *Escherichia coli* β-galactosidase were obtained from Sigma Chemical Ltd. (Darmstadt, Germany), Sialyl-methylumbelliferyl α-glycoside (MU-Neu5Ac) and 5-bromo-4-chloro-3-indolyl-α-D-*N*-acetylneuraminic acid (X-Neu5Ac) were purchased from Toronto Research Center (Toronto, Canada). 4-Nitro blue tetrazolium chloride (NBT) was obtained from Roche (Germany). *Vibrio cholera* sialidase was obtained from Behring-Werke (Marburg, Germany). All other chemicals were of analytical reagent grade from Sigma Chemical Ltd. (Darmstadt, Germany).

Polyacrylamide gel electrophoresis

SDS-PAGE (Laemmli, 1970) was performed on a 10 and 7 % of slab polyacrylamide gel for *Vibrio cholera* sialidase and *Escherichia coli* β-galactosidase, respectively, using BioRad electrophoresis apparatus. 8 to 24 mU of sialidase and 2.2 to 6.5 U of β-galactosidase were mixed with electrophoresis sample buffer under non-reducing condition. After incubating about 5 min on ice, the enzyme preparations were loaded on the gels and were run at 100 volt (~ 20 mA/gel) and 150 volt (~ 30 mA/gel) for the stacking gel and the separated gel, respectively, at room temperature until the bromophenol blue reached the end of the gel.

Sialidase and β-galactosidase activity assays on gels

(a) Using chromogenic substrates: After performing electrophoresis, gel was washed in a humidity chamber with 0.1 M acetic acid buffer pH 5.5 and Tris-HCl buffer pH 7.3 for sialidase and β-galactosidase assay, respectively. The buffer was refreshed 3 times in 10 minutes. A piece of filter paper was put on the base of the chamber and the gel was put on the filter paper. After that, the buffer was removed, however, the filter paper was kept wet to keep chamber humid. For the enzymes assay, a piece of filter paper, which has been soaked in chromogenic substrates, 0.2 mM X-Gal (pH 7.3) and 1.86 mM of X-Neu5Ac (pH 5.5), each containing 10 mg/mL NBT, for β-galactosidase and sialidase activity test, respectively, was put on the gel. The chamber was closed and incubated at 37 ^{o}C. The reaction for β-galactosidase was stopped after 10-30 min by addition of ethanol:acetic acid:water solution (30:10:60), while for sialidase a longer incubation time was needed (3-5 h). The substrate-containing filter paper was removed and blue color band detected was corresponded to the enzyme activity. The gels could then be stained for protein staining.

(b) Using fluorigenic substrates: After performing electrophoresis, the gel was prepared as described in the section of assay with artificial

chromogenic substrates above. However, the filter paper put on the gel was first soaked in 0.2 mM of MU-Neu5Ac (pH 5.5) and 0.2 mM of MU-Gal (pH 7.3) for sialidase activity and β-galactosidase activity test, respectively. The chamber was closed and incubated at 37 ^{o}C. The reaction for β-galactosidase was stopped after 1-3 min by addition of glycine buffer, pH 10.7, and the substrate-containing filter paper was removed. For sialidase, a longer incubation time (1-2 h) was needed. The gel was then illuminated with low UV light (366 nm). The fluorescence band appeared was correspond to the protein containing enzyme activity and the gels could then be stained for protein staining.

Results and Discussion

Using chromogenic substrates, X-Neu5Ac and X-Gal for sialidase and β-galactosidase, respectively, we demonstrated that these substrates were suitable for enzymatic staining in polyacrylamide gel. On the polyacrylamide gel electrophoresis of 10 %, a single blue band correspond to sialidase activity were determined (Figure 1b), while two blue bands correspond to the β-galactosidase activity was detected on the polyacrylamide gel electrophoresis of 7 % (Figure 2b). The same results were showed when enzymatic staining of sialidase and β-galactosidase was performed using fluorigenic substrates, MU-Neu5Ac and MU-Gal, respectively. Figure 1a and Figure 2a show the fluorescence bands correspond to the sialidase and β-galactosidase activity, respectively.

The following protein staining showing that the blue bands from the chromogenic substrates were identical to the 83 kD of *Vibrio cholera* sialidase preparation (Figure 1c), while the *Escherichia coli* β-galactosidase were detected on the two protein bands over 200 kD (Figure 2c).

The blue-indigo dye was visualized within the gel following desialylation and degalactosylation of X-Neu5Ac and X-Gal by the enzyme reaction, respectively. The sharped blue-band visualized within the gels was independent to the time incubation, since the indigo dye did not diffuse. The blue sharp band would be stronger with the longer incubation time or when NBT was applied (Michal *et al*, 1983).

The described enzymatic staining using

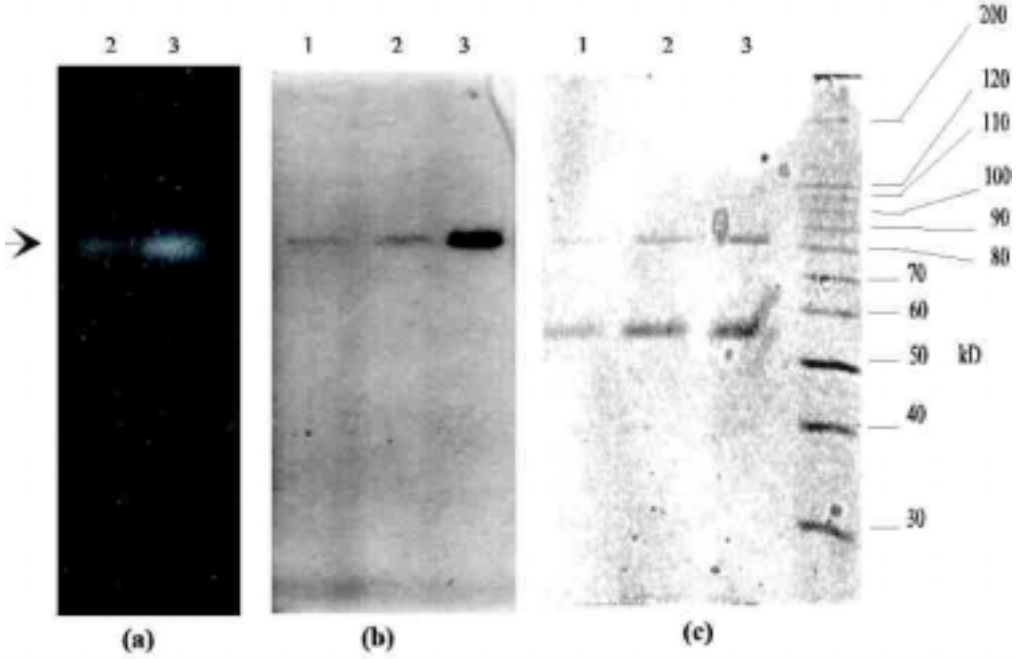

Figure 1. SDS-PAGE under non-reducing condition of *Vibrio cholerae* sialidase.
Sialidase activity is showed by the arrow. Lane 1, sample of 10 μL containing 8 mU sialidase; lane 2, sample of 20 μL containing 16 mU sialidase; lane 3, sample of 30 μL containing 24 mU sialidase. The sialidase applied has a specific activity of 2 U/mg. (a) fluorigenic staining, (b) chromogenic staining, and (c) coomassie brilliant blue staining.

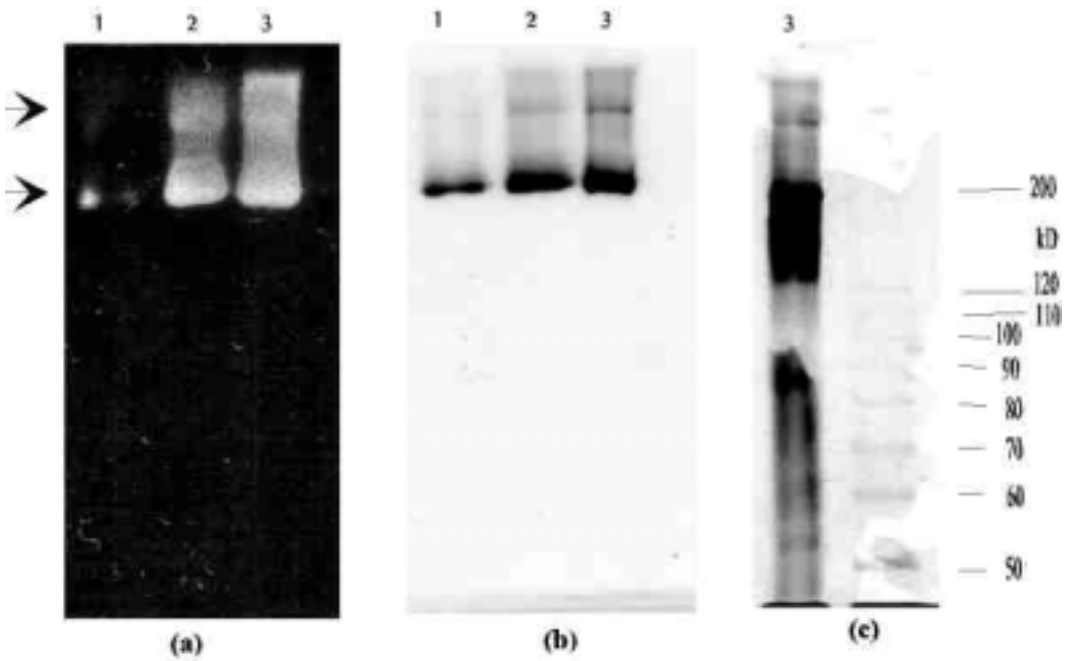

Figure 2. SDS-PAGE under non-reducing condition of *Escherichia coli* β-galactosidase type IV. β-Galactosidase activity is showed by the arrow. Lane 1, sample of 10 µL containing 2.2 U β-galactosidase; lane 2, sample of 20 µL containing 4.5 mU β-galactosidase; lane 3, sample of 30 µL containing 6.5 mU β-galactosidase. (a) fluorigenic staining, (b) chromogenic staining, and (c) coomassie brilliant blue staining.

chromogenic substrate is a useful tool to determine protein band correspond to sialidase or β-galactosidase. Furthermore this technique more convenience than using fluorigenic substrate, since the indigo dye resulted by the enzymatic reaction did not diffuse and be permanently stained within the polyacrylamide gel as in the case of 4-metylumbelliferone when MU-Neu5Ac was used. However for sialidase activity determination, a long incubation time was needed, since sialidase acted very slow to the X-Neu5Ac.

As mentioned by Berg *et al.* (1985), the sharpness of the fluorescence band depends on the incubation time, since the 4-methylumbelliferone diffuses rapidly, we recommended that the gels was checked for the fluorescent each 1 and 5 minutes for β-galactosidase and sialidase, respectively. Once a sharp fluorescent band was detected, a glycine buffer (0.133 M glycine buffer, pH 10.7, containing 0.06 M NaCl and 0.04 M Na_2CO_3) should be added to intensify the fluorescence (Potier *et al.*, 1979). Particular in the detection of the enzymes activities, fluorigenic substrates gave a faster test than chromogenic substrates. Using sialidase with the activity of 24 mU the fluorescence band (from the MU-Neu5Ac) could be seen after 10 minutes, while the indigo dye (from the X-Neu5Ac) emerged after 60 minutes.

In this such experiment, it is very important first to check the enzyme activity in the presence of electrophoresis sample buffer (Laemmli, 1970), because there is sialidase/β-galactosidase which is very sensitive to the electrophoresis sample buffer due to the presence of SDS (Candra *et al.*, 2000).

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